TARGETING IRON SEQUESTERATION: A NOVEL STRATEGY TO INHIBIT BACTERIAL PATHOGENS

A SYNOPSIS

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1. INTRODUCTION:

Though antibiotics have revolutionized the world of medicine, they started to pose a strong force on pathogens to evolve with new and quick strategies to counteract. Over a period of time the pathogens evolved with various counteracting strategies which lead to a condition called antimicrobial resistance (AMR). AMR occurs when bacteria, viruses, fungi and parasites change over time and no longer respond to medicines making infections harder to treat and increasing the risk of disease spread, severe illness and death (WHO). Nation-wide data obtained for *Escherichia coli*, *Klebsiella pneumoniae* and *Staphylococcus aureus* showed that the proportion of resistant to commonly used specified antibacterial drugs exceeded 50% in many settings (WHO, Antimicrobial resistance: global report on surveillance, 2014). Resistant bacteria especially Staphylococci*, K. pneumoniae, Pseudomonas spp.*, and enterococci are becoming harboured in healthcare institutions. AMR of these pathogens frequently results in treatment failure, which generates serious consequences, mainly in critically ill patients. Bacteria develop resistance towards antibiotics by various mechanisms such as efflux pumps, producing enzymes that degrade antibiotics and alteration of the targets [1].

Since AMR is becoming a global threat, it is crucial to find alternative approaches to control microbial infections. Various approaches were investigated by researchers as alternatives to antibiotics such as passive immunization [2], phage therapy [3], bacteriocins [4], probiotics [5] and tannins [6]. Targeting the pathways involved in iron-uptake in pathogenic bacteria is a novel and alternative strategy to antibiotics [7, 8].

Iron is an important nutrient for all bacterial species that infect human beings. Iron deprivation in the medium showed inhibition in the growth of bacteria [9]. Bacterial pathogens must therefore overcome the iron limitation mechanisms of the host to establish an infection. For this, they craft various tactics and strategies during the course of evolution. One of the mechanisms they use to sequester iron from the iron-bound host proteins is siderophore-based iron acquisition system. Siderophores outcompete the host proteins in binding the ferric ions because of their astounding iron-binding affinity. Siderophores are synthesized under low-iron availability and secreted out to

the extracellular environment, where they reach the ferric iron or chelate ferric iron from the iron-bound host proteins.

Many researchers have reported that there is a strong correlation between iron and the virulence of bacterial pathogens. The role of iron in virulence of bacterial pathogens is investigated by various researchers. Iron injections in rodent models of bacterial infection, enhanced the *in vivo* virulence of a large number of bacterial strains [10]. Microorganisms of limited pathogenicity such as *Yersinia enterocolitica,* usually causing mild enteric infection, may produce severe sepsis, intestinal perforation, or multiple abscesses in patients with transfusional iron overload or hereditary hemochromatosis [11, 12, 13]. Iron was also found to upregulate the expression of virulence genes in a number of bacterial pathogens [14]. These studies clearly indicate that interfering iron acquisition in bacterial pathogens could be a potential antimicrobial strategy.

For a past few decades, the routes used by pathogens to assimilate iron from the environment have been the subject of intense study, resulting in clear-cut molecular mechanisms of iron-uptake. This led to investigations on interfering with one of these iron assimilation pathways in pathogens as an approach to halt their growth. Though few investigators have tried this novel strategy of controlling pathogenic bacteria *in vitro*, this research is still in its infancy. Many more insightful investigations are needed in this area to develop novel drugs that can certainly be an alternative to antibiotics. Though this area of research is still naive, one of the earliest antimicrobials used for treatment of tuberculosis, *p*-aminosalicylic acid (PAS) was found to inhibit the biosynthesis of mycobactin [15], a siderophore produced by mycobacterium tuberculosis, which proves that the research on iron-uptake inhibitors, keeps moving in the right track.

Urinary tract infection (UTI) is the most common disease of the urinary system affecting urethra, bladder, kidney and urinary tract. UTI occurs due to colonization of bacteria, virus and fungi that may affect one or more parts of the urinary system [16]. UTI is also one of the most common nosocomial infections. It is usually classified into two types as un-complicated and complicated UTI predominantly affecting women than men. The pathogenic bacteria involved in uncomplicated UTI are *E. coli*, *K.*

pneumonia, *Staphylococcus saprophyticus, Enterococcus faecalis* and *Proteus mirabilis*. The bacterial species that are involved in complicated UTI are *Pseudomonas aeruginosa*, *Enterobacter spp.*, *Staphylococcus spp.* and *Acinetobacter baumannii* [17].

The current work focuses on the screening for novel molecules that target iron sequestration in *E. coli* and *P. aeruginosa* using a combination of *in silico* and *in vitro* methods. Also, this work attempts to investigate the role of iron in growth, virulence and pigment production in *P. aeruginosa.*

2. LITERATURE REVIEW

Bacteria are ubiquitous organisms dwelling in a wide environmental niche. Bacterial infections lead to a huge impact on public health and are transmitted to humans through various modes like contact, droplets, vector and airborne routes. The probability of infection by bacteria depends on the number of individuals infected with respect to the number of individuals exposed to the infection [18].

Bacterial adhesion is the preliminary stage of colonisation of bacteria and it is non-specific if bound to an inert surface and specific if bound to specific receptors [19]. Bacterial adhesion to host is mediated by adhesins, which are surface components capable of interacting with the extracellular matrix of host [20].

Biofilms

Biofilms are groups of microorganisms that are capable of adhering to a solid surface and proliferate into a three-dimensional structure with cells embedded as a micro-community within them. It helps in the protected growth of the microbes by ensuring their survival in harsh environmental conditions [21]. The cells attached to the biofilms are capable of producing extra-cellular polymer matrix, and many other compounds by selective regulation of certain genes. As a whole, a proficient biofilm would comprise of the environment with optimal conditions that would aid in the multiplication and nourishment of the cells [22]. It is found that biofilm production is a significant form of exhibiting virulence in many pathogens like *P. aeruginosa*, *S. aureus*, and *E. coli* for establishment of infection in the host.

Use and Overuse of Antibiotics

It is essential to control the pathogens in order to prevent the transmission of diseases and infection. Various antibiotics and chemotherapeutic agents are used to effectively control the pathogenic bacteria.

Antibiotics either inhibit the growth of bacteria or kill them outright based on their chemical nature and ability to inhibit various cellular processes of bacteria [23]. Despite the fact that the broad-spectrum antibiotics have been successfully used in the treatment of bacterial diseases, new and recurrent bacterial infections continue to impose significant challenges on global health care [24].

The vast majority of antibiotic use occurs in the outpatient setting, where up to a third of prescriptions are unnecessary. Plenty of studies indicate that antibiotics are often prescribed unnecessarily [25, 26, 27]. Almost 30% of children are prescribed with antibiotics during primary care visits [26]. Since antibiotics may kill the normal flora of the gut, there is a possibility of recurrence of the disease. In addition to that, inappropriate prescribing of antibiotics can lead to both drug-related adverse effects and the promotion of antibiotic resistance.

Antibiotic Resistance

Over-prescribing of antibiotics has arguably led to an epidemic of drug resistant microbes [28]. Thus, although antibiotic use may be beneficial to the individual, excessive use can be detrimental to the community. Antimicrobial Resistance (AMR) is established when microorganisms become able to adapt and grow in the presence of drugs that were originally used to control their growth [29]. The fact that infectious diseases can no longer be treated with antibiotics portrays an unknown future in health care [30]. AMR compromises not only the capacity of human immune system to defend against microbial infections, it also contributes to various complications in patients undergoing chemotherapy, dialysis, surgery and joint replacement.

Host Defense Against Infection

Combatting the threat of infectious diseases in order to establish a suitable surveillance mechanism for all the organs present in the body is performed by the network of cells and pathways associated with the immune system [31]. Innate immunity constitutes the first line of defence mechanisms of the body. The most common way of narrowing the innate immune response is performed by the crucial combination of the microbe and

host. The development of adaptive immune components is aided by the responses of innate immunity. Nutritional immunity is the phenomenon adopted by the host in which the nutrients necessary for the survival of the pathogen is collectively sequestrated to prevent the utilisation of it by the pathogen [32]. The host uses iron as a strategy of innate defence by maintaining iron in the complexed form with haem protein that is still enclosed within the erythrocytes. In case of availability of free haem, it quickly binds to other proteins like haptoglobin and haemopexin. Further iron is stored within the cell in the form of storage protein ferritin ensuring low accessibility to the pathogen. Usually, the ferrous ions present in the physiological pH are converted to insoluble ferric forms that binds to transferrin present in saliva, tears and breast milk with increased affinity [33].

Strategies by bacteria to overcome nutritional immunity:

Bacterial pathogens utilise numerous strategies to circumvent the barrier of nutritional immunity. Iron acquisition strategies adopted by the microbes are classified as those that are capable of developing systems for siderophores, haem and free ferrous iron. Siderophores are low molecular weight molecules produced by bacteria that are capable of chelating ferric ions with increased affinity than the proteins, transferrin and lactoferrin [32].

There are few receptors, present in the outer membrane of the bacterial cell, facilitate uptake of siderophores. These include: ferric enterobactin permease, ferric pyochelin transport, ferric pyoverdine, ferric vanchrobactin transport and ferric anguibactin transport [34]. The internalised siderophore is reduced so that the iron bound is converted to ferrous forms so that it could be utilised by the microbe. Then the unloaded siderophore is recycled for another round of iron uptake [35]. The types of bacterial siderophores present in bacteria include catecholate, trihydroxamate and mixed types with varying binding affinities for iron with respect to their ionic forms [36].

Targeting iron sequestration

As bacteria require iron for growth and for various functions including respiration and DNA synthesis, the pathways of iron assimilation are found to be attractive targets for the rational development of targeted therapeutics. The most superficial approach to control bacteria through perturbation of iron assimilation pathways is to directly compete with the bacterium for the nutrient. Under normal physiological conditions,

nutritional immunity is established by binding of free iron ions by transferrin and other iron-binding proteins. Since the siderophores are having high affinity towards iron, they are utilized in chelation therapy in modern medicine that will bind the free ferrous ions thereby creating an environment similar to the one created naturally by nutritional immunity.

Another approach is the utilization of iron assimilation pathways for treating infections ie. inhibition of molecules involved in the biosynthesis of siderophores. Various studies demonstrate the inhibition of siderophore production including but not limited to enterobactin, yersiniabactin, mycobactin, and acinetobactin which represent virulence factors of *E. coli*, *Yersinia pestis*, *Mycobacterium tuberculosis*, and *Acinetobacter baumannii*, respectively. Siderophore conjugates are being developed which act as a "trojan horse" to kill pathogens [37]. In this approach siderophores are coupled to toxic moieties that can be delivered to the periplasm or cytosol of bacterial strains. This class of compounds are also called as sideromycins. Two such drugs were produced against *Y. enterocolitica* namely salmycin and albomycin A1 [38].

3. OBJECTIVES:

- To isolate and characterize uropathogenic bacteria and screen for siderophore production.
- To study the influence of iron restriction on growth, virulence and pigment production in uropathogenic *P. aeruginosa*.
- Selection of drug targets involved in iron acquisition in *E. coli* and *P. aeruginosa* and screening for inhibitors.

4. RESEARCH PROBLEM:

With increasing prevalence of antimicrobial resistance in bacteria, it is crucial to find alternative strategies to control bacterial pathogens. One such strategies is inhibition of iron acquisition in pathogenic bacteria. Bacteria uses various strategies for iron acquisition one of which is by secreting iron chelating compounds called siderophores. Inhibition of biosynthesis and transport of siderophores is a novel strategy to inhibit pathogenic bacteria. High-affinity iron acquisition is mediated by siderophore-dependent pathways in the majority of pathogenic bacteria. Inhibition of siderophore mediated iron acquisition is one of the promising strategies to control bacterial

infections. Though inhibitors of siderophore biosynthesis and transport are reported earlier, still no approved drugs are available in the market. Similarly, limited or no studies are available on understanding the role of iron on growth, pigment production, virulence and biofilm formation in bacteria. This study attempts to explore the impact of iron on the growth, virulence and biofilm formation in uropathogenic bacteria.

5. ORGANIZATION OF THESIS

The thesis is organized into nine chapters, as presented below:

Chapter 1: Introduction

This chapter provides an introduction to the basis and requirement of the current research work. It explains how bacterial pathogens evolve with various strategies to combat the immune mechanisms exerted by the human body. The chapter highlights how antimicrobial resistance is becoming a threat to the humankind and why alternative strategies are required to control pathogens. The microorganisms causing urinary tract infections and the mechanisms of how they establish an infection are also discussed in the chapter. The chapter also explains the overview of the current work with observations and results.

Chapter 2: Literature Review

The chapter 2 analyzes the literature regarding various aspects of the study. Research works that investigated bacterial infections, mechanisms of infections, immunity against infections, nutritional immunity and the strategies used by pathogens to overcome the defense by human body were analyzed and quoted. The chapter also presents details about the mechanisms of iron acquisition by bacteria and the role of siderophores in it. Siderophores produced by uropathogenic *E. co*li and *P. aeruginosa* are also discussed in the chapter. It was found from the literature that little or no studies were found which investigated the role of iron restriction on growth, virulence and pigment formation in uropathogenic bacteria.

Chapter 3: Isolation and characterization of uropathogenic bacteria and screening for siderophore producers

Urinary Tract Infection (UTI) is the second most common bacterial infection in humans and thus represents a major source of human discomfort. About 50% of women are reported to experience UTI at some point in their lives (WHO). *E. coli* is the most

frequently isolated urinary pathogen accounting for 50 to 90% of all uncomplicated urinary tract infections. The chapter 3 describes the isolation of uropathogenic bacteria from urine samples from infected patients and also how they were screened for siderophore production. The urine samples were streaked onto differential medium that selectively allow the growth of Gram-negative bacteria and various biochemical tests were performed on the strains isolated from the plates. Screening for siderophore producers was carried out by streaking the isolates onto CAS agar plates and the siderophores produced were quantified by Arnow's assay. Siderophore produced by *P. aeruginosa* was quantified by fluorimetric analysis.

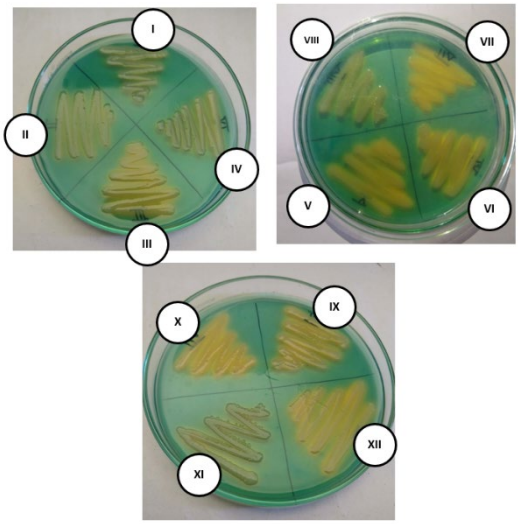

Figure 1: CAS assay for screening siderophore producing bacteria

Chapter 4: Influence of Iron Restriction on Growth, Virulence and Pigment Production in Uropathogenic *P. aeruginosa*

P. aeruginosa is considered to be a clinically important microorganism as it involves in variety of infections like urinary, respiratory, and other soft tissue related infections. The pathogen also possesses several defensive mechanisms against innate immunity as well as to antibiotics, where it reduces the intercellular drug concentration thereby reducing the dose required for the effective action against the pathogen. *P. aeruginosa* also contains some other mechanisms to safeguard them from host response and which includes decreasing membrane permeability and enzymes which will modify or degrade antimicrobials. *P. aeruginosa* was reported to cause complicated urinary tract infection. The most common virulence factors associated with the severity of the infection and

multidrug resistance in *P. aeruginosa* includes flagellum, pili, protein secretion systems, exoenzymes, lectins, siderophores, quorum sensing factors and biofilm matrix. *P. aeruginosa* produces three important pigments namely pyoverdine, pyocyanin, pyomelanin and pyorubin. The pigment pyoverdine acts as a siderophore, an iron sequestering agent and also reported to be one of the important virulence factors of *P. aeruginosa.* Limited or no studies are available on understanding the role of iron on growth, pigment production, virulence and biofilm formation in *P. aeruginosa*. This study attempts to explore the impact of iron on the growth, virulence and pigment formation in *P. aeruginosa* (Figure 2).

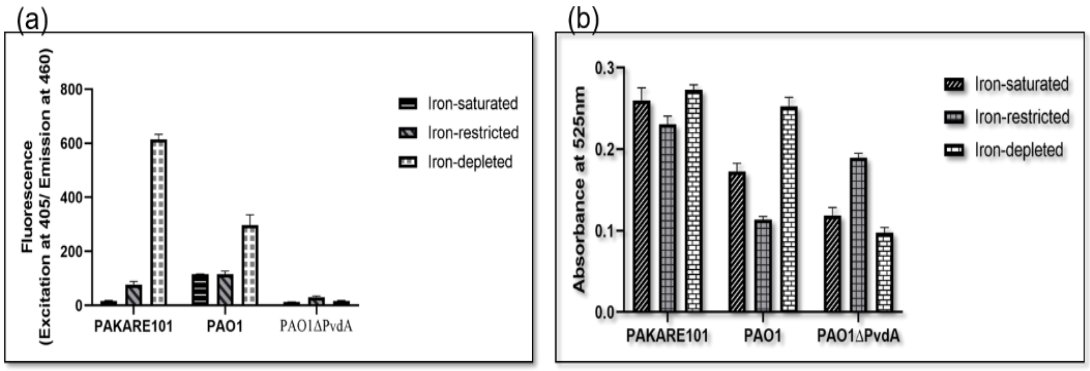

Figure 2: Role of iron in pigment formation in *P. aeruginosa*

Chapter 5: Selection of drug targets involving in iron acquisition in *E. coli* and *P. aeruginosa*

To inhibit iron-acquisition in bacteria, it is crucial to find proteins, enzymes or receptors which can be targeted using a small molecule drug. This work was performed to find novel drug targets involved in iron acquisition which can be specifically targeted with drug molecules. In order to find an effective drug target, it is important to know the molecular mechanisms of biosynthesis and transport of siderophores which are involved in iron sequestration in pathogens. Molecular mechanisms of enterobactin biosynthesis in *E. coli* and pyoverdine biosynthesis in *P. aeruginosa* were well studied and reported by the researchers. Isochorismate synthase (EntC), an enzyme involved in biosynthesis of enterobactin and, FepA, a bacterial cell surface receptor involved in the transport of enterobactin into the bacterial cells, were selected as drug targets for *E. coli*. L-ornithine monooxygenase (PvdA) and an acylase (PvdQ) were selected as drug targets for *P. aeruginosa* which are involved in biosynthesis of pyoverdine. The

characteristics of the drug targets were analyzed and various computational studies were performed to validate them as potential drug targets (Figure 3).

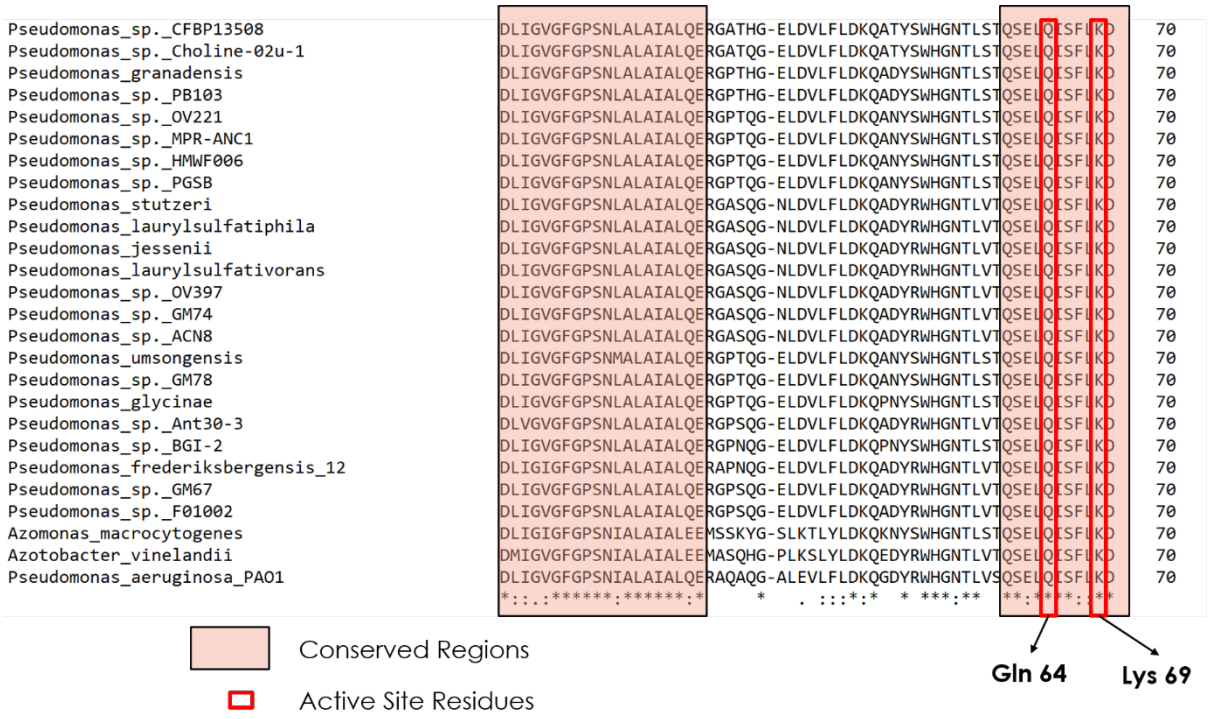

Figure 3. A part of multiple sequence alignment of FepA protein sequences from various microorganisms show that they are highly conserved

Chapter 6: *In silico* screening for inhibitors of iron acquisition in *E. coli:* Targeting ICS and FepA.

Enterobactin is a high affinity siderophore that acquires iron for microbial systems. It is primarily present in Gram-negative bacteria, such as *Escherichia coli* and *Salmonella typhimurium*. The biosynthesis and mechanism of transport of iron by enterobactin were well studied and reported by many researchers. An enzyme isochorismate synthase (ICS) involved in the biosynthesis of enterobactin and a membrane protein FepA, which is involved in the active transport of ferric-enterobactin complex from the extracellular space into the periplasm of Gram- negative bacteria were chosen as drug targets for this study. Inhibition of these target proteins may cease the survival and virulence of bacterial pathogens, which exclusively use enterobactin as iron chelators. In this chapter, a set of marine and plant derived metabolites were screened *in silico* as inhibitors of ICS and FepA (Figure 4). The active compounds which have high binding

affinity towards these targets can be purified and, further be studied as inhibitors of enterobactin biosynthesis and transport.

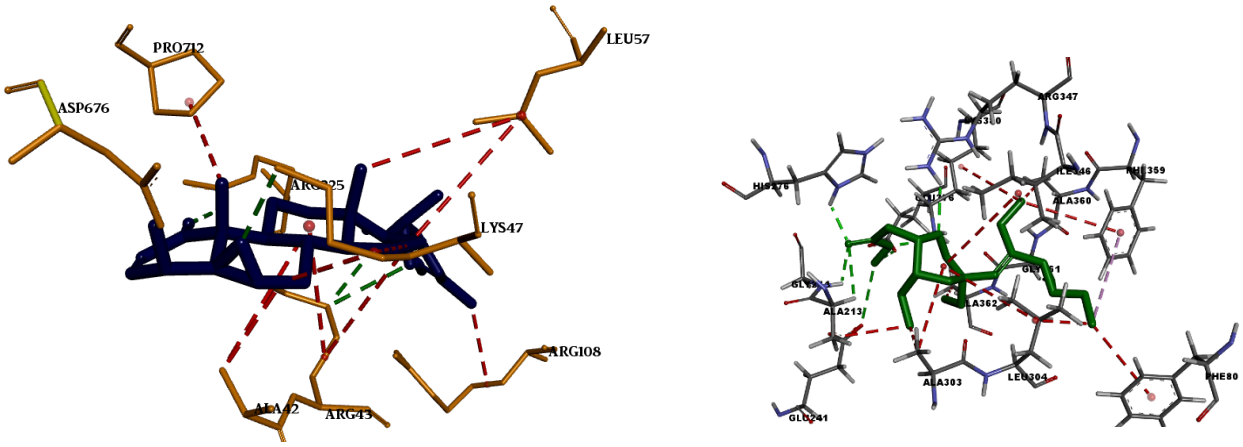

Figure 4: Interaction of natural metabolites with FepA (Left) and ICS (Right)

Chapter 7: *In silico* screening for inhibitors of PvdQ as a strategy to interfere iron acquisition in *P. aeruginosa*

As PvdQ, an enzyme involved in pyoverdine biosynthesis, impacts the adaptation of fluorescent pseudomonads to host surroundings, it has been considered as a target for inhibitors that optimistically may help to reduce the ability of *P. aeruginosa* to flourish in hosts. Later, having solved the structure of PvdQ from *P. aeruginosa*, researchers got ahead in identifying such inhibitors, which will be capable to inhibit development of *P. aeruginosa* under iron-limiting conditions. PvdQ belongs to the NTN hydrolase family. The members of the family are produced as inactive proteins and auto-proteolytically cleaved to yield active α/β-heterodimers. The N-terminus of the β–chain is preferably a serine that is the reason for auto-proteolysis and the catalytic cleavage of acyl esters. PvdQ was acknowledged as a periplasmic quorum quenching protein that cut acyl homoserine lactones. This chapter describes the research work that was focused on screening for inhibitors of PvdQ. Pharmacophore based database searching was carried out for finding novel compounds that could be developed into a potential inhibitor of PvdQ, which can thus inhibit the biosynthesis of pyoverdine (Figure 5).

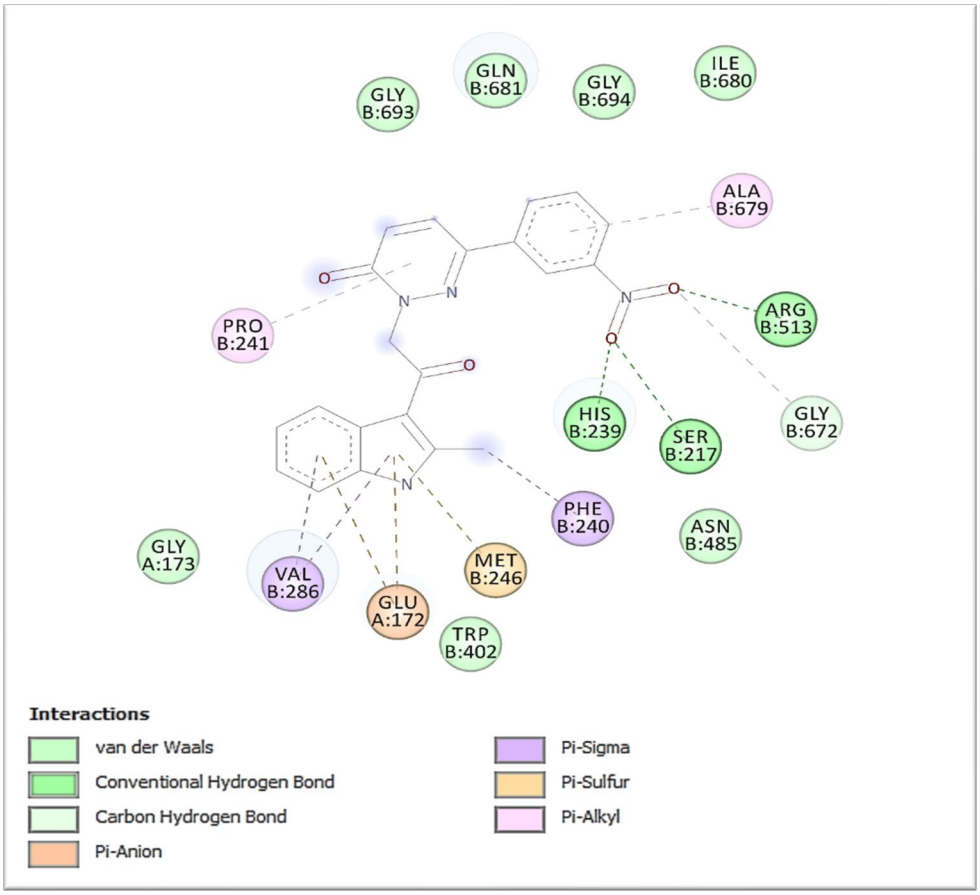

Figure 5: 2D Interaction diagram of PvdQ with the top binding compound 2-[2-(2-methyl-1*H*-indol-3-yl)-2-oxoethyl]-6-(3-nitrophenyl) pyridazin-3-one.

Chapter 8: *In silico* screening for inhibitors of PvdA as a strategy to target iron acquisition in *P. aeruginosa*

PvdA, involved in pyoverdine biosynthesis, is an attractive therapeutic target for controlling pseudomonas infections. Substrate analogue inhibitors are a class of antimicrobials that are successfully used for treating many infections and disorders. Though a few studies are available on searching for substrate analogues *in vitro*, to the best of our knowledge, no attempts have been made to find substrate analogue inhibitors using an *in silico* approach. Hence, the studies will lay the groundwork for exploring a novel method of designing substrate analogues *in silico*. The current chapter explains about the work that involve screening for substrate analogues that can bind to PvdA, an enzyme involved in biosynthesis of pyoverdine (Figure 6).

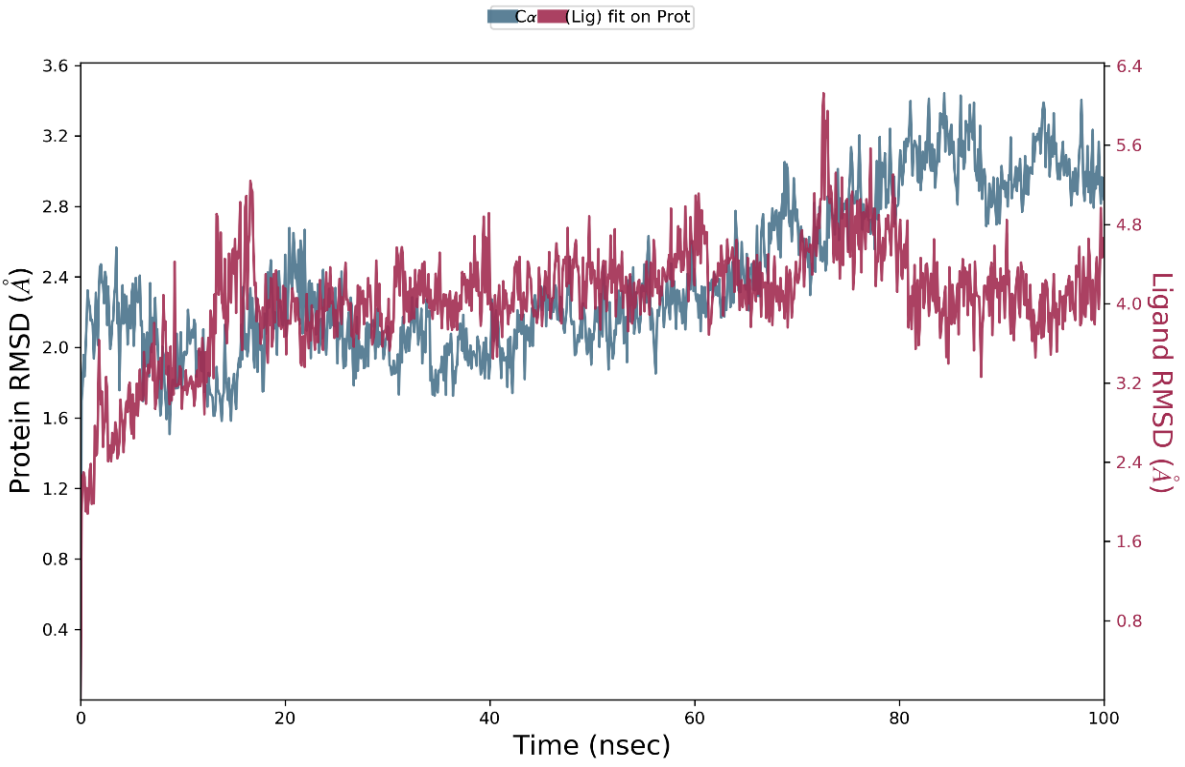

Figure 6: RMSD of protein and ligand throughout the 100 ns simulation of MD simulation

Chapter 9: Conclusion

This chapter summarises the findings of the current research work and explains how the results are in alignment with the literature. The study elucidates how iron modulates the growth, virulence and pigment production in uropathogenic bacteria. The *in silico* screening approaches demonstrated in this study opens a new direction of fast and low-cost screening for promising clinically effective molecules. The study also reports promising molecules which can be successfully developed into candidate drugs.

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List of Publications by the Candidate

1. **Christina Rosy, J**., E. Babkiewicz, P. Maszczyk, P. Mrówka, B.K. Kumar, S. Murugesan, S. Kunjiappan, K. Sundar (2022) L-Ornithine-N5-monooxygenase (PvdA) Substrate Analogue Inhibitors for Pseudomonas aeruginosa Infections Treatment: Drug Repurposing Computational Studies. *Biomolecules*, 12: 887. (**IF: 6.064**)

2. Rencilin, CF., **J.C. Rosy**, K. Sundar (2022) Generation of 2D-QSAR and pharmacophore models for fishing better anti-leishmanial therapeutics, *International Journal of Computational Biology and Drug Design* (Accepted for publication)

3. Mohan, M., P. Sivakumar, G.D. Dilip, **J.C. Rosy**, R. Coico, K. Sundar (2022) Computational analysis of proteome of Foot-and-mouth disease Virus for the prediction of immunogenic epitopes, *Vacunas* (Available online)

4. **Christina Rosy, J**., H. Ravinarayanan, P. Gokila, T. Navamuthumani, M. Shakti Chandra Vadhana, S. Kunjiappan, K. Sundar (2022) Virtual Screening of Natural Metabolites as Inhibitors of Biosynthesis and Transport of Enterobactin, *Biointerface Research in Applied Chemistry*, 13(12):125.

5. Rencilin, C.F., **J. Christina Rosy**, M. Mohan, R. Coico and K. Sundar (2021) Identification of SARS-CoV-2 CTL epitopes for development of a multivalent subunit vaccine for COVID-19. *Infection, Genetics and Evolution*, 89:104712, (**IF: 4.393**)

6. Marimuthu, S.C.V., H. Ravinarayanan, **J.C. Rosy** and K. Sundar (2021) Mining the Proteome of *Streptococcus Mutans* for Putative Drug Targets, *Infectious Disorders - Drug Targets*, 21(3):429-438. https://doi.org/10.2174/1871526520666200622143316

7. Ravinarayanan, H., **J. Christina Rosy**, R. Somavarapu, S. Ayswarya, B. Sundararajan, R. Subburaj and K. Sundar (2018) Anti-viral drugs against Ebola: A structure based virtual screening approach, *Ind. J. Biotechnol.*, 17(1): 176-184. (**IF: 0.324**)

8. **Christina Rosy, J**., S. Balamurali, J. Asnet Mary, R. Shenbagarathai and K. Sundar (2016) Generation of 2D- QSAR Model for Angiogenin Inhibitors: A Ligand-Based Approach for Cancer Drug Design, *Trends in Bioinformatics* 9: 1-13.